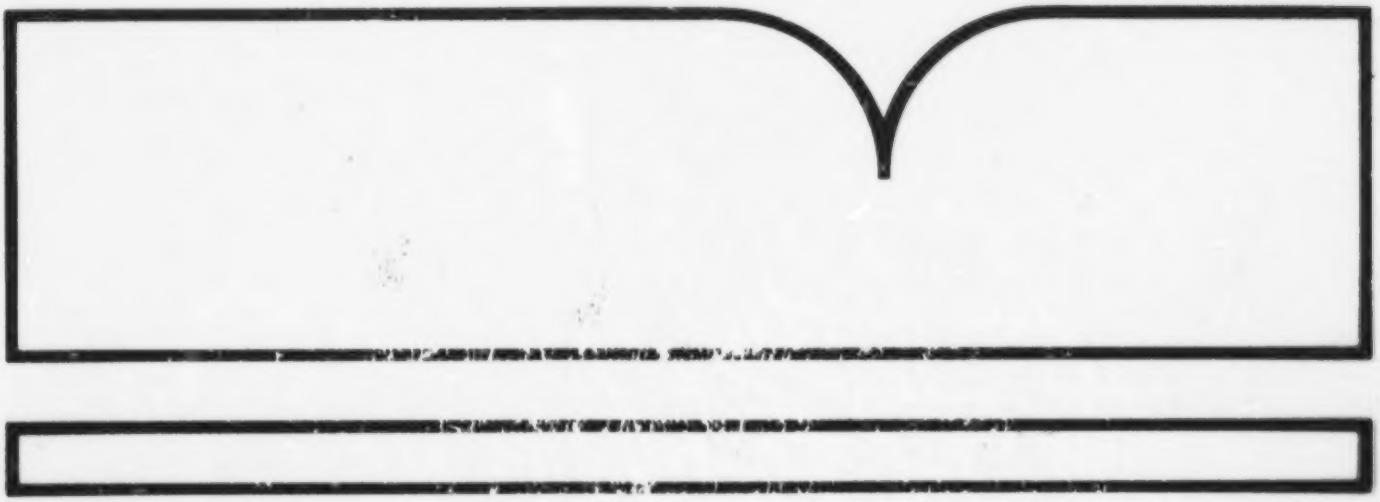
PB84-220920

Factor Interactions and Aquatic Toxicity Testing

(U.S.) Environmental Research Lab.
Narragansett, RI

1984



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1. REPORT NO.	2.	3. RE
EPA-600/J-84-045		PB84-220920

4. TITLE AND SUBTITLE	5. REPORT DATE
Factor Interactions And Aquatic Toxicity Testing (Journal Version)	1984
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
R.A. Voyer and J.F. Heltshe	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
U. S. EPA, ERL, Narragansett South Ferry Road Narragansett, RI 02882	AARB1A
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
U. S. EPA, ERL, Narragansett South Ferry Road Narragansett, RI 02882	Journal Article
	14. SPONSORING AGENCY CODE
	EPA/600/05

15. SUPPLEMENTARY NOTES

REF: Water Research, 18(4):441-447, 1984

16. ABSTRACT

Hypothesizing that experimental variables constituting an exposure situation act independently when in combination, we have reviewed two published data sets dealing with effects of metal mixtures on aquatic animals in order to assess the potential practical significance of factor interactions and their implication to the design of aquatic toxicity tests. Both data sets were re-analyzed using each of the following predictive models: (1) simple-additive, $y = a_x + b_y$, where y is an estimated response, a_x is the observed response to a concentration of toxicant x when $y = 0$, and b_y is the observed response to a level of toxicant y when $x = 0$; (2) linear-additive, $y = a + b_1x_1 + b_2x_2$, in which y is a predicted value. a and b are pooled estimates involving all treatments in the exposure assay; (3) quadratic response, $y = a + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$, which provides for estimates of interactions and non-linear effects. The relative effectiveness of each model in predicting joint effects of independent test variables was evaluated in terms of calculated mean-square error and goodness-of-fit (R^2) values, as well as by how well predicted treatment effects compared with responses observed by original investigators.

Our analyses show that in one case all three models provided similar estimates that closely approximated observed responses, despite the presence of a statistically significant two-factor metal interaction. In comparison, in the second instance, the quadratic response model was the most effective predictor and was appreciably better than the linear-additive model in terms of the calculated parameters. The simple-additive model on the other hand, tended to over-estimate treatment effects, by as much as 80% in some instances, and was least effective of the three models examined. Our re-analyses show that the working hypothesis is rejected, i.e. an assumption of factor independence is not to be accepted *a priori*.

A sequential testing protocol is presented which would permit an evaluation of the existence of factor interactions.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to Public	Unclassified	8
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (9-73)

EPA-600/J-84-045
JOURNAL ARTICLE

Water Res. Vol. 18, No. 4, pp. 441–447, 1984
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0043-1354/84 $3.00 + 0.00

FACTOR INTERACTIONS AND AQUATIC TOXICITY TESTING

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(*Received July* 1983)

Abstract—Hypothesizing that experimental variables constituting an exposure situation act independently when in combination, we have reviewed two published data sets dealing with effects of metal mixtures on aquatic animals in order to assess the potential practical significance of factor interactions and their implication to the design of aquatic toxicity tests. Both data sets were re-analyzed using each of the following predictive models: (1) simple-additive, $y = a_x + b_y$, where y is an estimated response, a_x is the observed response to a concentration of toxicant x when $y = 0$, and b_y is the observed response to a level of toxicant y when $x = 0$; (2) linear-additive, $y = a + b_1x_1 + b_2x_2$, in which y is a predicted value, a and b are pooled estimates involving all treatments in the exposure assay; (3) quadratic response, $y = a + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$, which provides for estimates of interactions and non-linear effects. The relative effectiveness of each model in predicting joint effects of independent test variables was evaluated in terms of calculated mean-square error and goodness-of-fit (R^2) values, as well as by how well predicted treatment effects compared with responses observed by original investigators.

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A sequential testing protocol is presented which would permit an evaluation of the existence of factor interactions.

Key words—heavy metals, factor interactions, predictive models, simple-additive model, linear-additive model, quadratic response model, experimental design

INTRODUCTION

The United States Environmental Protection Agency, by virtue of the Federal Water Pollution Control Act Amendment of 1972, the Toxic Substances Act, and the Clean Water Act of 1981, has the responsibility to evaluate potential effects of chemical substances discharged into the environment; and to develop water quality criteria reflecting effects of chemicals on biological processes in varying types of receiving waters. Accordingly, national water quality criteria (1980) have been prepared. Much of the data included in these criteria are from standard tests of single compounds using local laboratory waters, however. As a consequence, the general utility of these criteria is not clear, in that aquatic organisms in estuarine areas are more likely to encounter mixtures of toxicants than single poisons. Results of laboratory studies indicate that in the case of metals, for instance, two or more of these substances may have a joint effect that is simply additive as has been shown with Cu and Cd (Westernhagen *et al.*, 1979). Alternatively, joint effects may be either more-than-additive as in the case of Cu and Ag (Coglianese and Martin, 1981) or less-than-additive as with mixtures of Cu and Hg (Moulder, 1980) and some combinations of Pb and Hg (Gray, 1974). Furthermore, responses of estuarine animals may also be modified through the influence of environmental factors such as salinity and temperature (Jones, 1975; Sullivan, 1977; Hrs-Brenko *et al.*, 1977).

It is apparent that, as Westernhagen *et al.* (1979) have pointed out, the possible action of toxicants on aquatic organisms may become increasingly more complex and concomitantly less easily predicted as the number of factors available to interact increases. Interactions between toxicants and between toxicants and environmental parameters have been demonstrated to influence, at least statistically, a number of response parameters ranging from survival to rate of population growth (Gray, 1974; Nelson *et al.*, 1977; Voyer *et al.*, 1977; Roed, 1979). The degree to which these interactions may modify the usefulness of routine estimates of toxic effects of substances based on the use of standard laboratory test procedures is equivocal. The following effort was thus initiated in an attempt to assess the practical significance of factor interactions to the development of water qual-

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Contribution No. 348.

ity criteria and their implications to the design and conduct of laboratory aquatic toxicity tests.

HYPOTHESIS AND DATA REDUCTION

The working hypothesis used in this analysis is that experimental variables act independently when in combination, i.e. the toxicities of two or more hazardous compounds in a mixture are additive. To illustrate the concept of factor interaction two hypothetical examples are presented in Fig. 1. In both cases, responses are plotted on the ordinate as a function of effects of Compound **I** by itself and in combination with specific levels of Compound **II**. In both figures the bottom curve represents a response to Compound **I** alone. In the example of factor independence, the addition of Compound **II** results in changes in levels of responses that are uniform and consistent in nature as reflected by the series of parallel dose-response curves. This pattern of change contrasts with the one presented in the second example in which case the absence of parallelism is indicative of factor interaction, a differential response to one compound at various levels of a second.

If one were to attempt to predict changes in rate of responses due mixture effects from mean dose-response curves (bold dash lines) in each case, one could be reasonably successful in the example of factor independence. In the example of factor interaction, however over- and under-estimates of mixtures effects may result.

To test our hypothesis two data sets, that had been generated using factorial-type experimental designs, were taken from the literature and re-examined in terms of each of the three predictive models presented in Table 1. One deals with the inhibition of cell division of a ciliated protozoa, *Uronema marinum*, exposed to solutions containing chlorides of Pb, Hg and Zn (Parker, 1979). In this experiment, two strains of animals were tested: one which had been reared in a culture containing non-growth inhibiting levels of the three metals and a second one that had been cultured in uncontaminated media. Statistical analysis of experimental results by Parker indicated no significant differences in rates of cell divisions between the two strains when tested, although in the

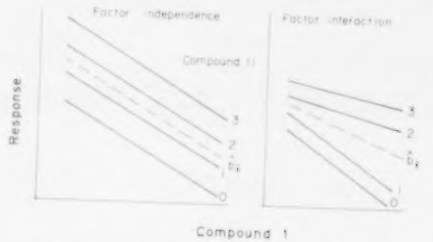

Fig. 1. Hypothetical dose-response curves for mixtures of two compounds (solid lines) and their relationship to a mean of all curves (dash lines) when factor interactions are either absent or present.

responses of the unacclimated strain there was more evidence of joint metal effects than in the case of the pre-exposed strain where only a Pb × Zn interaction was indicated. As a result we pooled the responses of both strains prior to our re-analysis.

The second set of data reviewed has to do with the viability of embryos of a marine teleost, *Pseudopleuronectes americanus*, exposed to mixtures of Cd and Ag at selected salinities (Voyer *et al.*, 1982). Regression analysis of these data indicated that each of the independent test variables influenced the percentage viable hatch, as did interactions between the two metals and between Cd and salinity. In this experiment Cd toxicity increased as salinity decreased. Silver *per se* was not toxic at concentrations tested, but when in combination with Cd it lessened the toxic effects of the latter metal.

Each data set was re-analyzed in terms of the three models presented in Table 1. The simple-additive model is the least sophisticated. Application of it involved simply summing observed responses at specific concentrations of each toxicant within a data set. For example, the normal development of an organism in a mixture of two hypothetical metals is 90% at $10\,\mu g\,l^{-1}$ of x when $y = 0$ and 70% at $10\,\mu g\,l^{-1}$ of y when $x = 0$. By addition, then, 40% of the organisms did not develop normally. Application of the simple-additive model would predict that the expected percentage of normal development in a mixture of x and y at these concentrations is 60%.

Predictions based on the linear-additive and quadratic models were made by re-analyzing each data

Table 1. Predictive models used in re-analyzing selected data sets

Simple-additive model:

$$\hat{y} = a_x + b_y$$

Linear-additive model:

$$\hat{y} = a + b_1x_1 + b_2x_2$$

Quadratic model:

$$\hat{y} = a + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$$

$\hat{y}$ = predicted response; a_x = the observed response to toxicant x when toxicant $y = 0$ and b_y = the observed response to y, when $x = 0$; x_1 and x_2 = independent variables; b_1, b_2 and b_{12}, b_{11} and b_{22} = estimated coefficients measuring linear, interactive and quadratic effects, respectively.

set using regression analysis techniques to calculate predictive coefficients for each model. Resulting equations were then used to predict a response at each of the original experimental treatments. The relative efficacy of each model was determined by comparing predictions with responses observed by original investigators. The more closely the predicted response approximated the observed for a given treatment combination the more effective the model was deemed to be. Model effectiveness was also judged in terms of calculated mean-square errors and goodness-of-fit (R^2) values. The smaller the mean-square error the better the model fits the data. The larger the R^2, on the other hand, the more closely the data fits the computed regression line.

RESULTS AND DISCUSSION

A comparison of mean-square error and goodness-of-fit values by data set and model (Table 2) suggests that for Parker's data all models reasonably represent test results. Seemingly, the Pb × Zn interaction suggested by statistical analysis was not sufficiently large that it could not be accounted for by the terms of the simple-additive model and the averaging effect of the linear-additive model. A similar comparison in the case of the Voyer *et al.* results indicates that these data are better represented the quadratic model with interaction terms than by either the simple-additive or linear-additive models.

The relative effectiveness of the three models is illustrated graphically in Figs 2 and 3 where differences between observed responses and those predicted by each model are presented. In the case of Parker's data (Fig. 2), it is apparent each of the three models did in fact predict comparable responses that closely approximated those observed at each treatment. For each exposure combination all predictions are within 20% of the observed response with most within 10%. Also, the Pb × Zn interaction suggested by statistical analysis is not discernible leading to the conclusion that it did not alter responses to a practical degree. This interpretation corresponds to the conclusion drawn by Parker that the effects of metal mixtures tested were essentially additive.

On the other hand, with regards to the Voyer *et al.*, data, inspection of Fig. 3 shows that at 10‰ salinity the quadratic model was the best predictor of mixture effects. The simple-additive model was least effective and the linear-additive model was intermediate in predictability. Further inspection of this figure shows also that differences in model predictability evident at 10‰ decreased as interactions diminished concomitant with increases in the experimental salinity.

It is noteworthy, too, that at 10‰ the simple-additive model tended to over-estimate mixture effects, demonstrating that it was not able to account for the factor interaction present resulting in a less-than-additive effect. In comparison, the linear-additive model yielded both over- and under-estimates of mixture effects. Under-estimates are, however, of a larger magnitude signifying that this model, in general, over compensated for the effect Ag had on Cd toxicity.

The inadequacy of the two latter models at 10‰ salinity can also be demonstrated by plotting responses (Fig. 4) recorded at this salinity in a manner comparable to that used in presenting the hypothetical case of factor interaction. The lowest curve in Fig. 4 represents the dose-response to Cd when Ag is absent, i.e. the independent effect of Cd. Comparison of it with those representing responses to Cd at various levels of Ag serves to illustrate further how the simple-additive model over predicted Cd effects in the presence of Ag. The bold dash line in this Figure denotes the average dose-response predicted by the linear-additive model. Comparison of this curve with others, as above, reveals how the linear-additive model under- and over-estimates mixture effects at low and high concentrations of Ag, respectively.

The two data sets reviewed serve to illustrate each of the two hypothetical instances presented. Parker's data correspond to the example of factor independence (Fig. 1) and show how joint effects of two or

Table 2. Predictive equations based on linear and quadratic regression models and data sets taken from literature. MSE (Mean square error) and correlation coefficients (R) determined using each model are included.

Reference	MSE	R^2
Parker (1979)		
Simple-additive model:	63.12	0.77
Linear model:		
$\hat{y} = 1.7394 + 10705.56Hg^* + 2.1792Pb^* + 0.5396Zn^*$	41.04	0.86
Quadratic model:		
$\hat{y} = -4.7502 + 14745.83Hg^* + 3.3332Pb^* + 0.8558Zn^* - 1130555.6Hg^2 - 0.0337Pb^2 - 0.0009Zn^2 - 31.250HgPb - 81.1458HgZn - 0.0392PbZn^*$	34.19	0.89
Voyer *et al.* (1982)		
Simple-additive model:	597.50	0.27
Linear model:		
$\hat{y} = 37.73 - 0.1049Ag + 0.0340Cd^* - 1.525Sal^*$	407.25	0.53
Quadratic model:		
$\hat{y} = 39.916 - 0.326Ag^* + 0.108Cd^* - 3.710Sal^* + 0.001Ag^2 + 0.000Cd^2 + 0.078Sal^2 - 0.000AgCd^* + 0.010AgSal^* - 0.004CdSal^*$	135.72	0.86

*Statistically significant variable at $P = 0.01$.

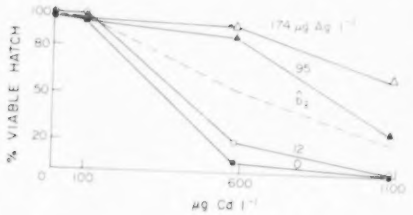

Fig. 4. Dose-response relationships showing percentage viable hatches of winter flounder embryos (*Pseudopleuronectes americanus*) exposed to various mixtures of cadmium and silver at 10‰ salinity and the relationship of each to a mean curve having an overall slope ($\bar{b}$).

medium may have contributed to the deviation from additivity.

SUGGESTED PROTOCOL

We conclude that, when attempting to evaluate the possible effects of toxicants on aquatic animals, an independence of factors constituting the exposure situation should not be assumed without supporting evidence. On the contrary, the presence of other possible lethal agents, together with those environmental parameters that may influence the chemistry of these agents and the physiology of test organisms need to be considered in designing test protocols. We submit that one of the first questions to be asked is, do toxicants and/or environmental factors interact? The most effective way of addressing the issue of factor interactions when using laboratory tests is to employ one or more experimental designs that permit direct evaluation of them. Factorial designs represent such a category. For the initial screening experiment a 2^k design is suggested. In this design, k factors are investigated at each of two levels. By using toxicant concentrations of absent and present, together with selected levels of environmental factors that may be of concern, this design insures that in a preliminary experiment the number of treatments is restricted to a manageable number and provides evaluation of interaction effects of factor combinations.

Levels of toxicants selected can be based upon dose-response information available or upon those concentrations anticipated in a given area. In using this design, selected toxicant levels need to be lower than what would be expected to affect 50% of the organisms treated, otherwise 100% mortality could occur in those treatments with several toxicants and mask interactive effects that may be present. If no evidence of interaction is found, the test material can then be evaluated using standard testing procedures currently in use (ASTM, 1980).

If, however, interactions are apparent, one might wish to generate a predictive equation that could be used to describe inter-relationships among variables over a range of both sublethal and lethal concentrations of mixture constituents and to interpolate responses within the ranges of exposure concentrations. Although full factorial designs can be used to generate information on interactive effects, central composite designs, as developed by Box and Wilson (1951) and described by Alderdice and Thomson (1974), provide a more economical means of evaluating the potential impact of several factors applied simultaneously. For example, a study of 4 variables using a 3^4 factorial design (4 factors each at 3 levels) would require 81 treatment combinations. In comparison, a composite design necessitates the use of 31 treatments. As noted by Alderdice and Thomson both designs provide the same amount of information per observation with the same relative precision. This design, together with multiple regression techniques, provide a powerful tool for determining functional relationships between dependent and independent variables and in generating response surfaces illustrative of these relationships (Myers, 1971).

Acknowledgements—We wish to thank Dr Tudor Davis for initial discussions, Drs G. Pesch and P. Rogerson for their constructive reviews, L. Lassiter, M. Brennan and P. Bussiere for their typing skill.

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